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ENDOGENOUS NITRIC OXIDE IS PRESENT IN THE EXHALED AIR OF RABBITS, GUINEA PIGS AND HUMANS

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The presence of nitric oxide (NO) in the exhaled air of humans and of anaesthetized rabbits and guinea pigs was demonstrated by chemiluminescence, diazotization and mass spectrometry. This NO is endogenously produced in the lung by an NO synthase, since its generation in guinea pigs and rabbits was inhibited by N^{ω}-nitro-L-arginine methyl ester and N^{G}-monomethyl-L-arginine, inhibitors of this enzyme. The effect of the inhibitors was reversed by the precursor of NO synthesis, L-arginine. Since NO is produced by normal vascular endothelium for the physiological regulation of blood flow and pressure and also by activated macrophages to contribute to non-specific immunity, our experiments suggest that NO may play both vascular regulatory and host defence roles in pulmonary physiology and pathophysiology. © 1991 Academic Press, Inc.

Recently, nitric oxide (NO), synthesised from the amino acid L-arginine, has been identified as the transduction mechanism of the soluble guanylate cyclase and also as an immunologically-derived effector molecule. In the vasculature, NO acts as a physiological regulator of blood flow and blood pressure whereas when synthesised by activated macrophages and other cells in the immune defence system, it plays a role in non-specific immunity, pathological vasodilatation and host tissue damage (1). The lung is an organ with an active blood/air interface responsible for the exchange of O_2 and CO_2. It also plays a role in the metabolism of endogenous vasoactive substances (2) and in immunological reactions via the alveolar macrophages and other cells (3,4). In the lung vasculature, NO has already been implicated in the modulation of the pulmonary circulation (5) and of the vasoconstriction which follows hypoxia (6,7,8). In view of this, and of the fact that NO might also modulate bronchial smooth muscle tone where a soluble guanylate cyclase is present (9,10), it was of interest to know whether NO itself in its gaseous phase was excreted in the expired air of animals and humans.

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MATERIALS AND METHODS

Rabbits of either sex were anaesthetized with sodium pentobarbital 60 mg kg^{-1} i.v., tracheotomized, paralyzed with pancuronium bromide i.v (1 mg kg^{-1}), and ventilated by a Harvard rodent ventilator (6 ml kg^{-1} $breath^{-1}$ at 40 insufflations min^{-1}). Adult male Dunkin-Hartley guinea pigs were anaesthetized with pentobarbital (60-80 mg kg^{-1} i.p.), tracheotomized and maintained on a ventilator, and one carotid artery and jugular vein were cannulated for blood pressure recordings and i.v. infusions. For rabbits and guinea pigs, expired gas was sampled from an open-end reservoir tube immediately after the ventilator exhaust valve and the gas taken either into chemiluminescence apparatus or collected by bubbling through trap solutions. In human experiments, the subject was asked to breathe continuously by inhaling through the nose and exhaling through a 7 mm i.d. reservoir tube, sampling air via a side arm of the reservoir.

Chemiluminescence: NO/nitrite in solutions was determined by chemiluminescence (11) by injecting samples and standards into a reaction vessel containing 100 ml 1% sodium iodide in concentrated acetic acid. The NO formed was carried by a flow (100 ml min^{-1}) of N_2 gas via a cold trap into a reaction chamber and reacted with ozone (100 ml min^{-1}). The sample-ozone reaction was quantified by a photomultiplier tube and continuously recorded on an ordinate writer. The detection limit, as nitrite, was 5 pmol as determined by linear regression. Nitric oxide gas measurements were obtained by allowing 100 ml min^{-1} sample gas to enter via the instruments N_2 inlet port, bypassing the reaction vessel. Calibration was performed by a certified gas (250 ppm NO in N_2, AGA, Lidingö, Sweden) by dilutions with precision flowmeters (AGA and Rotameter Flow Control, Vällingby, Sweden). The detection limit and resolution for NO in gas was below 2 ppb (parts per billion, vol/vol).

Trapping experiments: Nitric oxide in expirate from rabbits was trapped by bubbling through 300 ml double distilled water with 3 mg l^{-1} iron (II) sulfate and 3 mg l^{-1} sodium bicarbonate. After lyophilization, the formed nitrite was analyzed by chemiluminescence (above) or by diazotization (12). Nitric oxide gas from guinea pigs was trapped as nitrosothioproline (13) by bubbling through 10 ml fresh, degassed, aqueous thioproline (15 mM). Nitrosothioproline was extracted into 5 ml of ethyl acetate, taken to dryness under N_2, and derivatized by 50 µl of N-methyl-N-(tertbutyldimethylsilyl) trifluoroacetamide in 50 µl acetonitrile. 2 µl samples were injected onto a 10m x 0.32 mm CP-Sil-5-CB column interfaced to a Quattro mass spectrometer operating in positive chemical ionization mode with isobutane. Nitrosothioproline had a retention time of 4.76 mins (He 30 cm sec^{-1}; 80°C for 3 min rising to 280°C at 40°C min^{-1} and was monitored at its pseudomolecular ion (m/z 277).

RESULTS

Nitric oxide, as detected by the chemiluminescence method, was excreted in the expired gas (Fig. 1 & 2) both in anaesthetized rabbits (at 15 ± 0.8 parts per billion (vol/vol), mean ± SE for $n=18$, $p<0.001$) and in the human (8 ± 0.8 ppb, $n=5$, $p<0.01$). The NO was unaffected by passing through a cold trap at -80° C, and showed no discernible breakdown over a 4 min delay coil, but was absorbed by a column of activated charcoal.

The production of NO could also be demonstrated by capture onto iron (II) sulfate and subsequent quantification of the formed nitrite by chemiluminescence or diazotization. As determined by diazotization 12 pmol min^{-1} was captured in expirate from a rabbit breathing a mixture of 20% O_2 in N_2. Sham trapping with the same gas ventilated into a gas-tight balloon yielded

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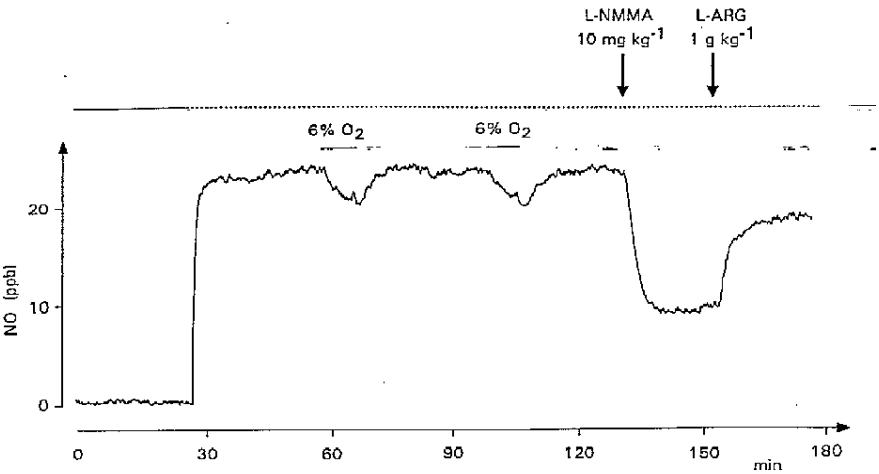
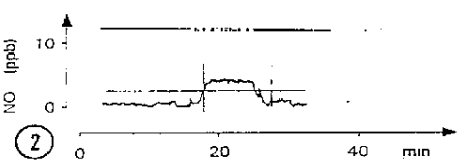


Fig 1. Detection, by chemiluminescence, of NO in expired air from anaesthetized rabbit ventilated with synthetic air (20% O_2 in N_2). Solid horizontal line indicates measurement on inspired gas, dotted line indicates measurement on expired gas. NO measured as vol/vol fraction in parts per billion (ppb). Decrease during hypoxia (6% O_2 in inspired air). Inhibition by i.v. N^G-monomethyl-L-arginine (L-NMMA) 10 mg kg^{-1} and reversal of this effect by i.v. L-arginine (L-ARG 1 g kg^{-1}).



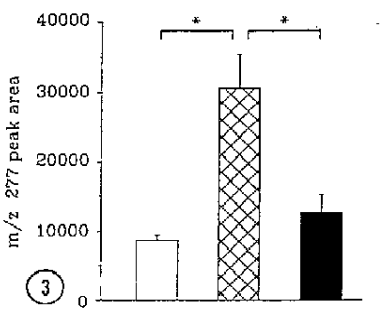


Fig 2. Detection of nitric oxide by chemiluminescence measurement on expired air from spontaneously breathing normal human subject. Solid horizontal line indicates measurement on inspired gas (room air), dotted line indicates measurement on expired gas. The measurement was obtained at time of minimal urban activities, yielding constant background levels below 1 ppb.

Fig 3. Detection of NO in expired air from guinea pigs as measured by gas chromatography-mass spectrometry of nitrosothioproline resulting from the trapping of NO by thioproline (see Methods). Quantification was performed by measurement on the pseudomolecular ion at relative mass number 277 by means of a Quattro mass spectrometer in positive chemical ionization mode, during elution of derivatised nitrosothioproline from a CP-Sil-5-CB column, expressing peak area in arbitrary relative units. Open column indicates sham sampling from room air (n=3), cross hatched column denotes measurement on expirate from untreated guinea pigs (n=4), and filled column denotes measurement on expirate from guinea pigs treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1} i.v. plus 30 mg kg^{-1} i.p., n=3). * denotes $p<0.05$.

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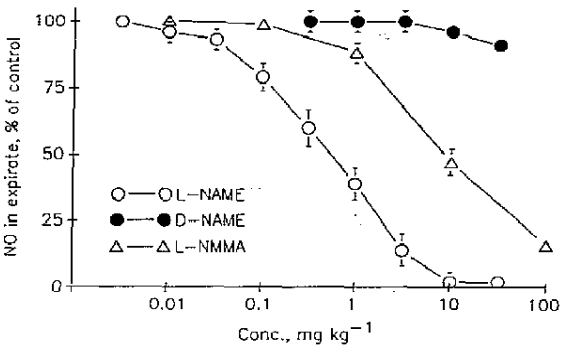

Fig 4. Rabbits in pentobarbital anesthesia, ventilated at constant volume (8 ml kg^{-1} breath^{-1} at 40 breaths min^{-1}) with a synthetic athmosphere containing 20% O_2 in N_2. Cumulative logarithmic dose response curves for i.v. N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-nitro-D-arginine methyl ester (D-NAME) or N^{G}-monomethyl-L-arginine (L-NMMA) on NO in expired air as measured by chemiluminescence. Mean values ± SE, n=3-8 except for 10 and 30 mg kg^{-1} D-NAME where n=1.

5 pmol min^{-1} of nitrite. The recovery (1-2%) of the trapped NO was similar as when sham-ventilating with comparable NO mixtures in synthetic air. The presence of NO in expirate was also demonstrated by nitrosation of thioproline and subsequent analysis by gas chromatography-mass spectrometry of the formed nitrosothioproline (Fig. 3). The formation of product was significantly ($p<0.05$) greater when sampling from expired air of guinea pigs ($n=4$) than from room air ($n=3$).

To study whether the expired NO was produced from L-arginine, the NO synthase inhibitors N^{G}-monomethyl-L-arginine (L-NMMA) or N^{ω}-nitro-L-arginine methyl ester (L-NAME) were injected intravenously in rabbits. L-NMMA at 10-100 mg kg^{-1} markedly inhibited and L-NAME at 10-30 mg kg^{-1} abolished NO in the expired gas (Fig. 1 & 4). The effect of L-NMMA (Fig. 1) or L-NAME at doses of up to 10 and 1 mg kg^{-1} respectively were partly or fully reversed by L-arginine (1 g kg^{-1} i.v.), whereas higher doses were only moderately reversed. D-NAME was virtually inactive (Fig. 4) and D-arginine failed to reverse effects of inhibitors (not shown).

In guinea pigs, L-NAME (10 mg kg^{-1} i.v. followed by 30 mg kg^{-1} i.p.) also significantly ($p<0.05$) inhibited the NO in expired air as measured by trapping and subsequent gas chromatography-mass spectrometry (Fig. 3). In these experiments, mean blood pressure was 52 ± 2 mm Hg, and increased to 80 ± 2 mm Hg by administration of L-NAME, remaining at this elevated level throughout the gas collection period. Administration of L-NAME had no effect on pulmonary insufflation pressure of the guinea pigs.

The effect of brief periods (5-10 min) of hypoxia on expired NO was studied in rabbits ($n=6$). Weak effects were seen at an inspired concentration of 14

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and 10% O_2 (not shown), whereas at 6% O_2 reproducible (Fig. 1) and significant ($p<0.05$) decrements in expired NO were obtained. The NO level in the expired gas remained, and in fact increased, after blood circulation was stopped by i.v. injection of air or He, and the sensitivity to changes in inspired O_2 remained. This suggests that the NO in the expirate derives from the lung or airways, and that the NO-producing cells depend largely on airway or alveolar oxygen.

DISCUSSION

The present study demonstrates that endogenous NO is excreted into expired air. This could be demonstrated in three different ways, namely, by chemiluminescence (11) of the expired gas and by detection of captured NO as nitrite by the diazo method (12) and by mass spectrometry (13). Of the methods used the chemiluminescence gives direct evidence for the presence of NO gas in the expirate since bypassing the reflux vessel for gas measurements in the system precludes the possibility of measuring NO_2^-. Furthermore, the amounts of NO_2^- measured by diazotization after the conversion of NO are consistent with the direct measurements of the gas. The formation of nitrosothioproline from thioproline by the L-arginine:NO pathway has been documented (13), although the precise mechanism for this nitrosation is unknown. The fact that specific inhibitors of the NO synthase inhibit the formation of nitrosothioproline strongly supports the above evidence for the presence of NO in the exhaled air. Furthermore, the inhibitors of the NO synthase inhibited the appearance of the NO dependent chemiluminescence and this was reversed by L-arginine, both effects being stereospecific.

A role for NO in the pulmonary circulation and in the normal oxygenation of the blood through regulation of ventilation-perfusion matching in vivo has recently been suggested (5). This is supported by the changes in NO concentrations induced by changing the levels of O_2 in inspired air. At this stage, it is not possible to ascertain whether the NO is formed by a constitutive or an inducible NO synthase (see 1), however, it is highly probable that for this physiological purpose a Ca^{+2} dependent NO synthase is involved in the generation of NO.

In addition, and/or alternatively, exhaled NO might be formed by macrophages and other cells in airways or in the alveolar surface. Indeed, these cells are known to be activated as a result of the retention of antigens and particles in the inhaled air (3,4), and induction of an NO synthase is one feature of the activation process of macrophages (1).

In summary, our results demonstrate that endogenous NO is present in exhaled air. The exact origin as well as the functions of this NO remain to be determined, but it is likely that as in many other organs several cell types will

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synthesise NO for physiological as well as pathological purposes, such as in vascular regulation and in host defence processes.

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